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The Isoniazid Paradigm of Killing, Resistance, and Persistence in *Mycobacterium tuberculosis*

Catherine Vilchèze, William R. Jacobs Jr.

Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1301 Morris Park Avenue, Bronx, NY 10461, USA

Abstract

Isoniazid (INH) was the first synthesized drug that mediated bactericidal killing of the bacterium *Mycobacterium tuberculosis*, a major clinical breakthrough. To this day, INH remains a cornerstone of modern tuberculosis (TB) chemotherapy. This review describes the serendipitous discovery of INH, its effectiveness on TB patients, and early studies to discover its mechanisms of bacteriocidal activity. Forty years after its introduction as a TB drug, the development of gene transfer in mycobacteria enabled the discovery of the genes encoding INH resistance, namely, the activator (*katG*) and the target (*inhA*) of INH. Further biochemical and x-ray crystallography studies on *KatG* and *InhA* proteins and mutants provided comprehensive understanding of INH mode of action and resistance mechanisms. Bacterial cultures can harbor subpopulations that are genetically or phenotypically resistant cells, the latter known as persisters. Treatment of exponentially growing cultures of *M. tuberculosis* with INH reproducibly kills 99% to 99.9% of cells in 3 days. Importantly, the surviving cells are slowly replicating or non-replicating cells expressing a unique stress response signature: these are the persisters. These persisters can be visualized using dual-reporter mycobacteriophages and their formation prevented using reducing compounds, such as *N*-acetylcysteine or vitamin C, that enhance *M. tuberculosis*' respiration. Altogether, this review portrays a detailed molecular analysis of INH killing and resistance mechanisms including persistence. The phenomenon of persistence is clearly the single greatest impediment to TB control, and research aimed at understanding persistence will provide new strategies to improve TB chemotherapy.

Keywords

isoniazid; persister; mycobacteriophage; mechanism

The Continuing Global Health Problem of Tuberculosis

Tuberculosis (TB), a disease caused by the bacillus *Mycobacterium tuberculosis*, remains the 10th leading cause of death and the single greatest cause of death from an infectious agent in the world [1]. It is estimated that one-fourth of the world's population is infected

Correspondence to William R. Jacobs: william.jacobs@einstein.yu.edu.

Declarations of Interest

None.

with *M. tuberculosis* [2,3], and in 2017, there were 10 million new cases of TB, and 1.6 million people died of TB [1]. Children are the primary victims of this disease, with one million of becoming sick and nearly a quarter of a million ultimately succumbing to death in 2017 [3].

TB is a curable disease that necessitates long treatment duration with multiple drugs. Chemotherapy for drug-susceptible TB uses the four first-line TB drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) for the first 2 months of treatment followed by 4 months on INH and RIF [4]. During this extensive treatment duration, drug resistance might develop due to patients interrupting their therapy prematurely, drugs of poor quality, or incorrect drug prescriptions [3,5,6]. Drug-resistant TB was reported in every country with documented cases of TB [7]. In 2016, there were more than half a million new TB cases resistant to RIF, and 82% of these cases were also resistant to INH [7]. Treatment of TB that is co-resistant to INH and RIF, also called multidrug-resistant TB, is complex and requires the use of a cocktail of drugs, with severe side effects, for as long as 2 years [3,8,9].

On September 26, 2018, the United Nations General Assembly declared its commitment to fight to end the TB epidemic by 2030, citing multidrug-resistant TB as a critical global health challenge. Although government officials and health care providers have the primary roles in fulfilling the United Nations' goals in ending the TB pandemic, researchers must understand and conquer the diverse ways *M. tuberculosis* has developed to escape drug action and the immune system, mechanisms that have allowed the organism to continue thriving among the human population. In this article, we will focus on the first-line TB drug INH, from its discovery, to its mode of action, and the mechanisms *M. tuberculosis* has exploited to evade INH killing.

The Origins of Chemotherapy and Antibiotics and the Need for Multidrug Therapies

TB has not only been the historical focal point of microbiology but also one of the great frustrations of antibacterial drug development. Arguably, Robert Koch's presentation on the "Aetiology of Tuberculosis" was the birthplace of microbiology as he clearly established the germ theory of disease. Prior to his studies, most thought TB was a genetic disease or a cancer. As Koch wrote: "To prove that TB was caused by the invasion and multiplication of bacilli, it was necessary to: isolate the bacilli, grow it in pure culture, and demonstrate that transmission to an animal caused the same morbid conditions as the original disease" [10].[†] One of the attendees at that famous lecture was Paul Ehrlich, who commented that the lecture was the greatest lecture he had ever heard. As an expert in dyes to stain immunological cells, Ehrlich established a collaboration with Robert Koch to improve staining of tubercle bacilli. Ehrlich reasoned that, since bacteria could be stained specifically, it should be possible to develop drugs to specifically kill them. Thus, Ehrlich provided the first description of chemotherapy [11–13], and his group went on to discover

[†]Many textbooks refer to this statement as "Koch's postulates," but logic would call it a singular postulate that the tubercle bacillus causes a disease following the fulfillment of three conditions.

salvarsan, the first drug used to treat bacterial infection, namely, syphilis [14]. Gerhard Domagk continued to build and screen the Ehrlich chemical library and discovered sulfonamides [15,16] as new drugs to treat bacterial infections.

As opposed to chemical libraries, Alexander Fleming made the discovery that a compound from *Penicillium notatum*, namely, penicillin, could kill bacteria [17]. Penicillin, even to this day, remains one of the greatest discoveries in infectious disease research, yet penicillin fails to have any activity against tubercle bacillus. In 1943, David Schatz discovered streptomycin, a natural product that could actually kill tubercle bacilli [18]. Unfortunately, 85% of the patients treated with streptomycin developed streptomycin-resistant TB in the first clinical trial of streptomycin [19,20]. The addition of paraaminosalicylic acid (PAS), discovered by Domagk [16], could reduce the emergence of streptomycin resistance [21,22], establishing the first multidrug therapy [19]. We argue that the phenomenon of persistence, the phenotype that allows *M. tuberculosis* to generate drug-resistant mutants [23–25], necessitates the need for such combination therapy.

The Discovery of Isoniazid: Serendipity and Rational Drug Discovery

The word “serendipity” was first coined in 1754 by Horace Warpole and was based on a Persian fairy tale entitled “The Three Princes of Serendip.” In this story, the heroes made their discoveries by accident and sagacity [26], and serendipity accurately reflects the way many discoveries are made in science, with INH being a perfect example. While trying to test whether nicotinamide, a component of niacin (vitamin B₃), could alleviate the side effects of ionizing radiation on cancer patients, Ernest Huant discovered that some of his cancer patients, who were also infected with *M. tuberculosis*, had dramatically cleared their TB lesions [27]. In parallel, French physician Vital Chorine tested nicotinamide on *M. tuberculosis*-infected guinea pigs and found high doses (1 g/kg) of nicotinamide stopped disease progression. Nicotinamide activity was specific to mycobacteria and was not due to its vitamin activity [28]. The knowledge that niacin could have activity against TB led Herbert Fox at Hoffmann–La Roche to screen compounds that resembled the niacin structure (Fig. 1), ultimately resulting in the discovery of INH [29]. At the same time, two other pharmaceutical groups led by Domagk at Bayer and Bernstein at the Squibb Institute for Medical research reported the activity of INH against *M. tuberculosis* [21,22,30]. Remarkably, this was the first drug in history with superb and specific bactericidal activity *in vitro* and *in vivo* against *M. tuberculosis*.

Elucidating the Mechanism of Action of INH—Pre-Genes Transfer Technology

Although discovered in 1952, studies identifying the specific target of INH action, that is, the mycolic acid biosynthetic pathway, would have to wait until the 1970s. Studies before that time concluded that the INH mechanism of action involved inhibition of DNA biosynthesis [31], an NAD glycohydrolase [32–35], a pyridoxal-dependent metabolic pathway(s) [36], cell division [37], or a membrane component [38–46]. Winder and colleagues [47] were the first to demonstrate that INH inhibited the biosynthesis of mycolic acids, long-chain α -alkyl, β -hydroxy-fatty acids that are an integral component of the

mycobacterial cell wall. Later, Takayama and colleagues [48] explored the mechanisms behind mycolic acid inhibition by INH and found that INH inhibited the biosynthesis of very long-chain saturated (NC26) and unsaturated (NC24) fatty acids, resulting in the accumulation of long-chain (C26) fatty acids. They postulated that these fatty acids were the precursors of mycolic acids and proposed that INH inhibited one of three enzymes: (1) a desaturase, (2) a cyclopropanase, or (3) an enzyme involved in fatty acid elongation [48]. The elucidation of the exact mechanism of INH action would have to wait until the 1990s when gene transfer, DNA sequences, and recombinant DNA technologies became available for *M. tuberculosis*.

Elucidating the Mechanism of Action of INH—Post-Gene Transfer Technology

Knowledge of the mechanism of INH action and resistance began with the isolation of INH-resistant mutants, DNA sequence analysis of the genes involved, and transfer of putative resistance alleles to drug-susceptible strains to prove causality. While Cohn and colleagues [49] first isolated INH-resistant mutants in 1954 and discovered that a large subset of these mutants were defective in catalaseperoxidase functions [50–52], it was clear that catalase peroxidase could not be the target of INH and yet somehow conferred INH susceptibility. Saroja and Gopinathan [53] had isolated INH-resistant mutants in *Mycobacterium smegmatis*, but the lack of a genetic map made it difficult to sort out a putative target for INH. Following the first introduction of foreign DNA into mycobacteria using a shuttle phasmid [54], a systematic approach was used to isolate a mutant of *M. smegmatis*, named mc²155, that was transformable with plasmids [55]. An initial step to elucidate specific genes involved in INH resistance made use of the INH-resistant mutant of mc²155 named BH1, which had impaired catalase activity [56]. Transformation of an *M. tuberculosis* library into this mutant led to the isolation of an INH-susceptible clone. Further subcloning demonstrated that a single gene, *katG*, encoding a catalase peroxidase, restored INH susceptibility, not only in the INH-resistant *M. smegmatis* mutant but also in INH-resistant *M. tuberculosis* clinical isolates [57]. Biochemical analysis demonstrated that *KatG* cleaved the hydrazide group on INH to form an isonicotinoyl radical, which reacts with NAD⁺ to form an INH-NAD adduct (Fig. 1A) [58–60]. BH1 led to the discovery that INH was a pro-drug activated by *KatG*. Two other *M. smegmatis* mutants, mc²651 [61] and mc²2359 [62], would be needed to decipher the genetic target of INH.

Previous studies had demonstrated that INH-resistant strains isolated from TB patients were often ethionamide (ETH) resistant, although the patients had never received ETH. Based on the hypothesis that INH and ETH share a common target, Banerjee and colleagues [61] isolated an *M. smegmatis* mutant, mc²651, that was co-resistant to INH and ETH. A genomic library of mc²651 was constructed and transformed into INH-susceptible mc²155 to identify clones that gained co-resistance to INH and ETH. Subcloning and sequence analysis led to the identification of a single open reading frame that conferred co-resistance to INH and ETH, and that was consequently named *inhA*. DNA sequence analysis of mc²651 *inhA* revealed a single base-pair mutation resulting in the amino acid change Ser94Ala [61]. Later, a single open reading frame that conferred co-resistance to INH and

ETH, and that was consequently named *inhA*. DNA sequence analysis of mc²651 *inhA* revealed a single base-pair mutation resulting in the amino acid change Ser94Ala was identified as encoding an NADH-dependent enoyl-ACP reductase [63,64], part of the fatty acid synthase type II system [65]. This finding led to the conundrum of how the potential inhibition of an enoyl-ACP reductase, whose role is to reduce ²-alkenoyl-ACP into saturated acyl-ACP, by INH would result in the accumulation of long-chain fatty acids [66] as observed by Takayama and colleagues [48]. This dilemma was solved with the isolation of mc²2359 [62], a temperature-sensitive *M. smegmatis* mutant co-resistant to INH and ETH and carrying a mutation in *inhA* (Val238Phe). This mutant demonstrated that thermal inactivation of *InhA* resulted in cell lysis, inhibition of mycolic acid biosynthesis, accumulation of long-chain fatty acids, and cell wall morphology changes, thus replicating the features of INH inhibition in mycobacteria observed by Takayama and colleagues [48,67,68]. These discoveries allowed us to propose *InhA* as the relevant target of INH. Further genetic proof came later when the *InhA* Ser94Ala mutation was introduced into wild-type *M. tuberculosis* H37Rv using a specialized transducing phage carrying the mutation Ser94Ala in *inhA* linked to a hygromycin cassette [69]. Screening for hygromycin resistance allowed the selection of transductants that carried the mutated *inhA*, and these transductants were both INH and ETH resistant, demonstrating that the Ser94Ala mutation in *inhA* was sufficient to cause INH resistance in *M. tuberculosis*. Furthermore, the activated form of INH, that is, the INH-NAD adduct, was shown bound to *InhA* by x-ray crystallography [59] and to inhibit the enzymatic activity of *InhA* [70,71]. These findings meet the criteria for identifying the drug target as the enzyme *InhA*, to which the drug (INH) binds and causes enzymatic inhibition leading to cell death. Moreover, overexpression of *inhA* either via promoter mutation [69] or by introducing *inhA* on a multicopy plasmid [72] also conferred INH resistance in *M. tuberculosis*, fulfilling another genetic concept of a drug target in which overexpression of the target leads to drug resistance via titration of the drug.

Although mutations in numerous genes have been found in INH-resistant clinical isolates of *M. tuberculosis* [73], only mutations in *katG* and *inhA* have been correlated with INH resistance. The proportion of mutations in *katG* and *inhA* varies geographically, but it is estimated that at least 80% of all INH-resistant clinical isolates have either the mutated Ser315 codon of *katG* or the c-15 t nucleotide substitution in the *inhA* promoter [73–75]. Since its introduction as an anti-TB drug 66 years ago, numerous targets and mechanisms of action have been proposed for INH [76]. In our current view, INH is a pro-drug activated by *KatG* to form an INH-NAD adduct, which binds to and inhibits *InhA*, leading to *M. tuberculosis* cell death.

The Phenomenon of Persistence

The discovery of penicillin, the first antibiotic or drug that mediated bactericidal killing, unmasked a previously unappreciated mechanism of drug resistance described as persistence. In contrast to drug resistance, in which bacteria acquire a genetic alteration that allows for growth in the presence of the antibiotic, persistence reflects the ability of bacteria to survive the killing activity of a drug without mutation. The phenomenon of persistence was first described by Hobby and Meyer in 1942 [77]. The authors observed that penicillin killed 99% of streptococci present in a culture. The rest of the bacteria (1%) could be

eliminated but were killed at a slower rate than were the first 99%. Later, Joseph Bigger found that penicillin often failed to sterilize *Staphylococcus pyogenes* cultures due to the survival of a small number (less than 1 per million) of bacteria. Bigger [78] named that surviving population “persisters.” He concluded that (1) persisters are present in a small number in cultures; (2) persisters are randomly distributed in bacterial cultures; (3) persister formation could be induced by external stresses (temperature shock, culture dilution, penicillin addition); (4) some staphylococci might be predestined to become persisters; (5) persisters are in a dormant, non-replicating phase; and (6) when the drug stress is removed, the persisters grow normally and are as sensitive as the parental culture to the drug. In addition, Bigger postulated that persisters had to exist *in vivo*. Based on the observation that staphylococcal infection in patients treated with penicillin for 8 days seemed to be cleared for a few days before re-emerging, Bigger suggested that persisters, which could stay dormant for long periods of time in patients, were responsible for the re-emergence of the disease and that multidrug treatment could prevent their growth.

Persistence in TB was manifested when patients were treated with the first TB drug, streptomycin. Unfortunately, 85% of the patients treated with streptomycin developed streptomycin-resistant *M. tuberculosis* and renewed TB symptoms [20]. The addition of PAS seemed to reduce the number of cases that developed drug-resistant organisms but still did not eradicate *M. tuberculosis* in many TB patients. It was only after INH was implemented in the 1950s, in combination with streptomycin and PAS, that a reproducible sterilizing chemotherapy became available [79]. Walsh McDermott, who pioneered the use of INH in humans, went on to establish a mouse infection model that reproducibly generated persisting *M. tuberculosis* in *M. tuberculosis*-infected mice treated with INH or a combination of PZA (an INH analog, Fig. 1A) and INH [80]. McCune and colleagues [80] observed that chronically infected mice had culturable *M. tuberculosis* bacilli after a 79-day treatment only when treated with INH and not when treated with PZA or with the combination of PZA/INH. In the latter case, *M. tuberculosis* could be isolated in about a third of these treated mice 3 months after treatment cessation. The authors demonstrated that surviving bacilli were fully susceptible to drugs used during the treatment. McDermott and his group defined persistence as “the capacity of the tubercle bacilli to resist sterilization in mice tissues” [81]. This definition differs from Bigger’s description of the phenomenon, which clearly suggests that persistence is a property of the bacterium, whereas McDermott thought persistence in mice or man could reflect the inability of the drug to get to tissues in privileged sites within mammals.

Treatment of *M. tuberculosis* with INH (Fig. 1B) *in vitro* follows a killing kinetics mimicking a typical pattern of persister formation [82], with a rapid killing of drug-susceptible *M. tuberculosis* cells followed by a reduction in the killing rate. This biphasic killing of the bacilli by INH was also observed *in vivo* either in INH-treated *M. tuberculosis*-infected guinea pigs [83] or in TB patients [84]. In the latter study, INH monotherapy on TB patients resulted in a 0.722 log₁₀ decrease in colony-forming units (CFUs) per day during the first 2 days of treatment followed by only a 0.113 log₁₀ CFUs/day decrease from day 2 to day 14, demonstrating that INH activity was the greatest during the first 2 days of treatment and then dropped drastically. Although the authors did not postulate a reason for this radical change in activity, others have proposed that this change in killing rate reflects

the emergence of INH resistance [85]. In the former study [83], INH treatment of guinea pigs resulted in a four-log decrease in CFUs within 2 weeks followed by a twolog decrease in CFUs in the next 4 weeks. Since no INH-resistant mutants emerged in the INH-treated *M. tuberculosis*-infected guinea pigs, the authors concluded that the decline in INH killing was due to the presence of phenotypically tolerant persisters.

In a notable study entitled “The death and resurrection of the tubercle bacillus,” Loring and colleagues [86] described drug-sensitive *M. tuberculosis* that was isolated from patients following chemotherapy (INH and streptomycin) and that required prolonged culture times (up to 16 weeks) to grow, but once grown, the bacteria would grow normally, infect, and cause TB disease in animals. They concluded “The fact that bacilli which we ordinarily regard as being dead become alive again, and active, is a matter of major interest.”

Although we do believe that persistence can have unique phenotypes in the host, we pursued the development of an *in vitro* model described below to study *M. tuberculosis* persistence in the presence of INH [87], building on the models used by Bigger, Hobby, and colleagues for other bacteria decades ago.

Detecting INH Persister *M. tuberculosis* Cells

M. tuberculosis persisters have been hard to visualize and quantitate. They represent a very small number of bacteria, often in a dormant or slowly replicating state. To further amplify this hardship, an *M. tuberculosis* population in a stressed or dormant state, such as the persisters, can lose the ability to stain acid-fast [88–90], a hallmark of *M. tuberculosis* and an easy way to evaluate microscopically for the presence of tubercle bacilli in human samples.

We developed a phage-based system to visualize persisters, based on the transcriptomic analysis of the INH persister cells [87]. *In vitro*, INH rapidly kills 99.0% to 99.9% of INH-susceptible *M. tuberculosis* bacilli during the first 3 days of treatment (Fig. 1B, slope “a”). In the second phase (slope “b”), the killing rate decreases due to the presence of INH persisters tolerant to INH action (Fig. 1B). The 0.1% to 1% of surviving cells are clearly persisters as upon regrowth they are fully INH-susceptible and will elicit the same death kinetics in retreatment with INH. Transcriptomic analysis of the persister cells that accumulated at day 4 indicated that these cells were (1) INH-susceptible, (2) non- or slowly-replicating, and (3) adapting to stress in order to survive [87]. This data set highlighted specific genes that were up-regulated in the persisters, and we chose promoters of these genes to construct dual-reporter mycobacteriophages (DRMs) to specifically visualize and quantify *M. tuberculosis* persister cells. These mycobacteriophages expressed both mVenus (GFP) from the phage constitutive promoter P_{L(L5)} and tdTomato (RFP) whose expression was controlled by the promoters of genes up-regulated in INH persisters (Fig. 2A). One such DRM, Φ²DRM9, in which the expression of tdTomato is driven by the promoter of the chaperone gene *dnaK*, has been used to visualize persisters produced during drug treatment [87,91,92] or present in sputum samples of TB patients [87]. Time-lapse photography of *M. tuberculosis* cells infected with Φ²DRM9 on a microfluidic chip and incubated in INH-containing media revealed that the majority of cells initially displayed a greenish-yellow

fluorescence (Fig. 2B). Actively dividing, DRM-infected *M. tuberculosis* cells expressed GFP and RFP when subjected to a stress such as drug treatment, nutrient starvation, or other. However, at day 3, the yellow fluorescent cells went dark (consistent with INH killing of actively dividing cells), and approximately 1% of the cells stayed fluorescently red, consistent with an INH persister population, that is, a non- or slowly dividing population in which the L5 phage promoter, expressed in metabolically active cells, was turned off, and the *dnaK* promoter was highly expressed [87]; see the movie <http://mbio.asm.org/content/7/5/e01023-16/DC2/embed/inline-supplementary-material-2.avi>). Flow cytometry analysis of the populations at day 3 shows the INH-treated *M. tuberculosis* culture contains a significant portion of RFP⁺GFP⁻ cells most likely representing the INH persisters (Fig. 2C and in Ref. [87]). Our studies provide a new tool, the DRMs, to visualize and quantitate persister cells. As we are convinced that many different stresses can induce persister formation, unique DRMs might need to be generated for visualizing other types of heterogeneous persister populations.

Mechanisms of INH Persistence

Since persisters represent a heterogeneous population whose formation might depend on the means used to generate this population (i.e., nutrient starvation, hypoxia, drug treatment), deciphering the mechanism(s) by which persisters are formed is challenging. Mechanisms involving toxin–antitoxin systems, stress response, SOS response, reactive oxygen species (ROS) response, stringent response, energy metabolism, efflux pumps, (p)ppGpp network, quorum sensing, and biofilms have been proposed to explain the presence of persisters and are comprehensively reviewed [82,93–97]. We will present a few mechanisms relevant to the formation of INH persisters in mycobacteria.

INH persistence can be instigated by cells that do not respond to the killing action of INH. INH is bactericidal only when activated by *KatG*; therefore, *KatG* shutdown to prevent the formation of the INHNAD adduct might enable *M. tuberculosis* cells to persist during INH treatment. Niki and colleagues [98] demonstrated that *KatG* was negatively regulated in mycobacteria by the histone-like protein MDP1 (mycobacterial DNA-binding protein 1), a regulator of gene expression involved in the mycobacterial growth rate [99]. MDP1 expression was found to be higher in stationary phase, resulting in a decrease in *KatG* expression. The authors proposed that tolerance to INH could be growth-phase dependent and mediated by increased MDP1 expression. Furthermore, Wakamoto and colleagues [100] demonstrated that *KatG* expression consisted of stochastic pulses. Mycobacterial cells with nonpulsing *KatG* levels did not activate INH and therefore were impervious to INH killing. The authors postulated that *KatG* pulsing might result from (1) stochastic ability of *KatG* to be on or off, (2) network switches that turned *KatG* on or off, or (3) production of ROS, during metabolic respiration, that triggers intermittent *katG* transcription.

INH persistence is impaired in cultures of *M. tuberculosis* strains deleted for the sigma factors *sigE* or *sigB* [101]. Interestingly, the sigma factor σ^E regulates the transcription of *relMtb* [102] encoding the GTP pyrophosphokinase involved in the synthesis and degradation of the “alarmone” (p)ppGpp (guanosine tetra- or pentaphosphate) [103,104]. (p)ppGpp is viewed as a regulator of persistence in bacteria [105,106]. In *M. tuberculosis*,

deletion of *relMtb* results in a strain that does not produce (p)ppGpp [107] and is unable to survive nutrient starvation [107], anaerobic conditions [107], or in mouse lungs during the chronic infection stage [108] indicating that (p)ppGpp is involved in *M. tuberculosis* persistence. Another member of the σ^E regulon is the two-component system MprAB. Phosphorylation of MprA by MprB using the inorganic polyphosphate polyP activates mprAB resulting in the activation of *sigE* and ultimately *relMtb*, leading to (p)ppGpp production [102]. PolyP may therefore also play a role in persistence as a regulator of (p)ppGpp levels in mycobacteria. PolyP is synthesized by the polyP kinase encoded by *ppk1*, which is regulated by both σ^B and the RNA polymerase binding protein RbpA [109]. Wang and colleagues [109] found that INH-tolerant cells were decreased in an *rbpA* knockdown strain and in *ppk1* and *sigB* knockout *M. smegmatis* strains, and concluded that RbpA, SigB and PPK1 form a regulatory network that controls polyP levels and INH tolerance. In contrast, Bhaskar and colleagues [110] generated *M. smegmatis* deletions in *relA*, the *M. smegmatis* homolog of *relMtb*, and genes involved in polyP synthesis (*ppk1*, *ppk2*) and degradation (*ppx1**ppx2*) and found that neither a decrease in (p)ppGpp levels nor changes in polyP concentration induced INH persistence.

Respiration is also a regulator of persistence in *M. tuberculosis* [111]. Increasing *M. tuberculosis* oxygen consumption either via genetic means, such as deletion of the succinate dehydrogenase *sdh1*, or by addition of reducing agents, prevents *M. tuberculosis* from entering into a persister state [111]. Sdh1 is an enzyme found in both the TCA cycle regulating the growth of *M. tuberculosis* and the electron transport chain, which controls energy production. Deletion of *sdh1* results in a strain unable to control its respiration or to persist in stationary phase [111]. The addition of reducing agents to INH-treated *M. tuberculosis* cultures prevents the formation of INH persisters and results in sterilization of the cultures [92,112]. In both instances, the inability of *M. tuberculosis* to enter into a persistent phase was due to an increase in the reduced menaquinone pool, the main electron carrier in the respiratory chain, leading *M. tuberculosis* to keep on consuming oxygen and thus to stay metabolically active [92,111,112]. The concept of boosting the bactericidal activity of a drug by increasing respiration was also demonstrated in *Escherichia coli* and *Staphylococcus aureus* [113]. The side effect of increased respiration in *M. tuberculosis* is ROS generation, which is bactericidal to *M. tuberculosis* [92,112,114,115]. Based on the observation made by Wakamoto and colleagues, we can postulate that this increase in oxygen consumption keeps *KatG* expressed to fight potential ROS production and therefore allows for INH activation and *M. tuberculosis* killing.

Dhar and McKinney [116] found that disruption of the cytochrome ABC transporter *cydC* impaired the ability of *M. tuberculosis* to enter into a persistent state in INH-treated mice while having no growth or survival effect in untreated mice. Although the role of *cydC* in mycobacteria is still unresolved [117], it is possible, as mentioned by the authors, that *CydC* is involved in maintaining redox homeostasis, leading us to hypothesize that deletion or impairment of this gene might shift the menaquinol pool balance and increase oxygen consumption by *M. tuberculosis*.

Eliminating Persisters

Novel drugs to target and eliminate persisters are the holy grail of *M. tuberculosis* drug discovery. Numerous laboratories, both academic and industrial, have targeted persisters through high-throughput screening of diverse libraries, a topic extensively reviewed by Gold and Nathan [118]. These authors emphasized the challenges facing such a task as most screens are done in specific *in vitro* conditions that may not reflect formation of persisters in TB patients, but they also expressed optimism that collaborations between researchers from various backgrounds, in both academic and pharmaceuticals laboratories, might improve understanding of the physiology of persisters in humans and how to eradicate these cells.

We recently demonstrated that INH persisters can be eliminated from INH-treated *M. tuberculosis in vitro* cultures by the addition of specific reductants such as vitamin C [112] or *N*-acetylcysteine [92] (Fig. 3A). Co-treatment of an *M. tuberculosis* culture with INH and vitamin C or *N*-acetylcysteine resulted in sterilization of the culture, whereas INH-resistant mutants emerged in an *M. tuberculosis* culture treated with INH alone [92,112]. Using the DRM Φ^2 DRM9, which allows for the visualization and quantification of persisters (see above), we demonstrated that the addition of vitamin C or *N*-acetylcysteine drastically reduced the persister population (Fig. 3B and C). As described above, these reductants increase *M. tuberculosis* oxygen consumption and keep *M. tuberculosis* cells in a metabolically active state in which they continue to divide, are susceptible to INH action, and are therefore killed by INH, preventing or eliminating the formation of INH persisters [92]. Interestingly, Syal and colleagues [119] demonstrated that vitamin C inhibits (p) ppGpp synthesis in *M. smegmatis*, suggesting that vitamin C can exploit different mechanisms to prevent persister formation in mycobacteria. Although vitamin C and *N*-acetylcysteine could be used as adjunct therapy for TB as they are nontoxic and, in the case of *N*-acetylcysteine, already used in TB patients to help cope with TB drug toxicity, the quantity required to achieve culture sterilization *in vitro* (mM range) may not be applicable in TB patients. Nevertheless, these findings suggest that screening for compounds that increase *M. tuberculosis* respiration could provide a new path towards *M. tuberculosis* eradication. Moreover, DRMs may provide the means for high-throughput screening to detect compounds that prevent persister formation.

Conclusion

The study of the mechanisms of INH action and resistance exposed the complex role of drug activation, target over-expression, and target binding. INH is a pro-drug that binds to and inhibits *InhA*, an enzyme involved in the biosynthesis of mycolic acids, an essential mycobacterial cell wall constituent, leading to mycobacterial cell death. The discovery of INH bactericidal activity against *M. tuberculosis* provided a central component of multi-drug therapy to cure *M. tuberculosis*-infected individuals, yet, to this day, TB remains a significant killer in the developing world with the potential of giving rise to drug-resistant organisms that could initiate untreatable epidemics. It is fair to ask why, 136 years after the discovery of *M. tuberculosis*, the organism that initiated the germ theory of disease, TB is far from being eradicated, although sterilization chemotherapy and a vaccine exist. We

believe that the reason for this failure is the specific property of persistence, a phenotype that *M. tuberculosis* has mastered to survive killing assaults.

Louis Pasteur said “It is characteristic of science and of progress that they continually open new fields to our vision” [120]. The development of genetics for *M. tuberculosis* has given us with new eyes to decipher the mechanisms of INH action and resistance and to understand the problem of persistence. By studying the kinetics of INH-induced killing of *M. tuberculosis* cells, one population of persistent *M. tuberculosis* cells which exhibits phenotypic INH resistance was revealed. Harnessing the knowledge of the unique transcriptional profile of INH persisters led to the construction of dual reporter mycobacteriophages to visualize these persisters. Further analysis of the genes and biochemical pathways involved in generating INH persister cells will provide new clues to shortening TB chemotherapy. Research efforts focused on the sterilization of the tubercle bacillus will surely involve understanding and eliminating the persistence phenomenon.

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Abbreviations used:

INH	isoniazid
RIF	rifampicin
PZA	pyrazinamide
EMB	ethambutol
PAS	para-aminosalicylic acid
ETH	ethionamide
DRM	dual-reporter mycobacteriophage
ROS	reactive oxygen species

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Fig. 1.

Isoniazid (INH) structure and killing of *M. tuberculosis*. (A) INH structure, analogs, and active form. Ethionamide, nicotinamide, and pyrazinamide are INH analogs, and INH-NAD is the active form of INH. (B) Biphasic killing of *M. tuberculosis* H37Rv by INH. H37Rv grown in Middlebrook 7H9 supplemented with OADC, glycerol, and tyloxapol was treated once with INH (7.3 μ M) at day 0. Bacteria survival was followed by taking samples at different time points and plating for colony-forming units (CFU). Slope "a" represents the rapid killing of INH-susceptible *M. tuberculosis* bacilli, slope "b" illustrates the slowing down of the bacterial killing by INH as persister cells accumulate, and slope "c" shows the emergence of INH-resistant mutants.

Fig. 2. Use of dual-reporter mycobacteriophages to monitor persister cells. (A) Representation of the dual-reporter mycobacteriophage $\Phi^2\text{DRM9}$. *mVenus* (GFP) expression is driven by the constitutive $P_{L(L5)}$ promoter, and *tdTomato* (RFP) expression is driven by the promoter of a specific gene highly expressed in the INH persister population (*dnaK* in $\Phi^2\text{DRM9}$). (B) Time-lapse microscopy of *M. tuberculosis* infected with $\Phi^2\text{DRM9}$ and treated with INH (from Jain et al. [87], with permission from the publisher). (C) Flow cytometry analysis of *M. tuberculosis* treated with INH (7.3 μM) for 3 days and infected with $\Phi^2\text{DRM9}$ for 16 h. The RFP population was back-gated to show the distribution of GFP expression in these cells.

Fig. 3. Vitamin C and *N*-acetylcysteine enhance INH killing of *M. tuberculosis*. (A) Sterilization of *M. tuberculosis* H37Rv with the combination of INH/Vitamin C or INH/*N*-acetylcysteine. H37Rv grown in Middlebrook 7H9 supplemented with OADC, glycerol, and tyloxapol was treated once with INH (7.3 μ M), vitamin C (VitC, 1 mM) or *N*-acetylcysteine (NAC, 4 mM) at day 0. Bacterial survival was monitored by taking samples at different time points and plating for colony-forming units (CFU). (B) Flow cytometry analysis of *M. tuberculosis* treated with INH, INH/VitC, or INH/NAC for 3 days and infected with Φ^2 DRM9 for 16 h. The RFP population was back-gated to show the distribution of GFP expression in these cells.(C) % RFP⁺GFP⁻ population (gate is shown in panel B) representing INH persisters.